Anti-Genes: siRNA, Ribozymes and Antisense

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Abstract: Scientists have been working on strategies to selectively turn off specific genes in diseased tissues for the past thirty years. In the 1980's, oligodeoxynucleotides (ODNs) with unique chemistries were tested with model systems both *in vitro* and *in vivo* with varying degrees of success. In the 1990's, ribozymes with both antisense and catalytic properties were successfully introduced to the field. Ribozymes were shown to selectively knock down targeted genes in human tumors grown in mice but delivery issues for these therapeutic anti-genes limited their clinical utility. Short interfering RNA (siRNA) is currently the fastest growing sector of this anti-gene field for target validation and therapeutic applications. The siRNA field may have an opportunity to impact the clinic faster than antisense and ribozymes if the scientists can overcome the previous anti-gene limitations. Fortuitously, there have been a several developments involving the expansion of our genomic knowledge coupled with the rapid dissemination of disease genes by the digital revolution. This convergence of the knowledge of the human genome with the speed of digital communication will help facilitate swift changes in the detection and treatment of human illnesses. The anti-gene field is positioned to exploit this timely union of two distinct technologies. Anti-gene molecules have an opportunity to become a successful technology in understanding the human genome, as well as, enabling the development of efficacious gene therapy for human diseases in the near future. This review will characterize the advances in this field and address the challenges to the success of for the anti-gene technology.

Key Words: Short interfering RNA (siRNA), Antisense; Phosphorothioate oligodeoxynucleotide (ODN), Ribozymes, Non-viral Vectors, Viral vectors, Oncogenes, Gene Therapy.

INTRODUCTION

Advances in molecular genetics have introduced new tools for the manipulation of gene expression, which will improve our understanding of the molecular basis of human diseases. The anti-gene technology is a double edge sword; it can be used as a strategy of target gene validation for a specific biological pathway as well as a therapeutic molecule. This anti-gene technology for target validation has often been referred to as "Reverse Genetics," where genes can be knocked out to help understand and define their function in a critical or disease pathway. The therapeutic potential of "Gene Therapy" for this anti-gene technology is being explored in clinical trials using a broad array of targets in human disease, most notably cancer. The principal appeal of anti-gene therapy is its inherent simplicity. This is based on the hypothesis that antisense DNA or RNA hybridizes to the target RNA in complementary Watson-Crick fashion, thereby resulting in sequence-specific suppression of gene expression [1]. Why has this simple therapeutic concept not fulfilled its clinical expectations in twenty years? There are several issues that need to be surmounted before these anti-gene therapies will become efficacious in the clinic. Primarily, the delivery of a effective pharmacological dose of the anti-gene will be required to impact the specific disease tissue. In addition, the non-sequence-specificity and non-antisense activity will require a better understanding if target selectivity is to be demonstrated. This review will cover three anti-gene strategies, their challenges and opportunities for identifying novel disease genes and their therapeutic applications for treating these diseases.

A. ANTI-GENE STRATEGIES

1. Short Interfering RNA (siRNA)

As with any new technology, siRNA has faced a large number of skeptics during the past fifteen years. Most plants and animal cells have unusual forms of RNA that can naturally inactivate gene expression. Understanding how these molecules function and learning how to manipulate these novel RNA molecules is still the challenge in mammalian cells [2]. At this stage, the siRNA technology has been validated in several plant and animal organisms but how it works and its therapeutic implications for human disease is still in progress.

It appears that double stranded RNA can be hydrolyzed into short sequences of RNA (interfering RNA; iRNA) by a cellular enzyme called the "Dicer". These 22 RNA nucleotide sequences are referred to as siRNA. The Dicer enzyme can cleave both strands of RNA leaving overhanging nucleotides on one strand at either end. The unwound single strand RNA, then can complex with the ribosome's to form an *RNA-induced silencing complex* (RISC). The selectivity of the reaction lies in the specificity on the 22 nucleotides and the target m-RNA. When the sequence match occurs, an enzyme known as the "Slicer" (siRNA) cleaves the target message in two. The RISC itself remains intact and is capable of renewing its catalytic activity against the target gene. Target genes have been inactivated by siRNA in a spectrum of organisms. The effect on the gene was rapid and persistent. The effects of siRNA can persist for days or

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weeks depending on the specific mRNA target, the cell type and the organism. In human cells, the studies have not been as dramatic as in the lower organisms that have been tested. These initial studies are very encouraging because of their high selectivity and a strong suppression of the target RNA [3]. However, as with any new technology, the limitations of antisense and ribozymes strategies are also the same limitations facing siRNA: the selectivity, delivery, and stability of double-stranded RNA species in mammalian cells represent significant obstacles that must be overcome prior to the maturation of this promising technology to the clinical arena.

2. Ribozymes

Self-cleaving RNA molecules were discovered in the late 1970's [4] and the publication on the trans-activating hammerhead ribozyme in the 1980's lead to a new set of tools to investigate gene expression [5, 6]. The therapeutic application of ribozymes has been studied from viral infections to cancer cells. The secondary structure of the hammerhead ribozyme has been well described [1, 6, 7]. The hammerhead ribozyme is composed of two regions: a catalytic core effecting cleavage and two flanking sequences confirming binding and specificity. The molecule confers specificity by the flanking sequence and the GUC cleavage site. The shorter the flanking sequences (6-10 nucleotides for each flanking sequences) confer a more rapid turnover rate. The catalytic core has a consensus cleave target sequence of XUN. X is any nucleotide and N is only A, C, or U nucleotides. The preferred target sequences contain the sequences of GUC, GUA, GUU, CUC or UUC [5-7].

The hammerhead ribozyme can inhibit target gene expression by several mechanisms that need further investigation. The ribozymes have both catalytic activities as well as binding capacity to the RNA, in addition to activation of RNase degradation through recognizing of the double stranded RNA [8].

3. Antisense Oligodeoxynucleotides (ODNs)

Antisense molecules are commonly composed of single-stranded DNA, where they are termed antisense oligodeoxynucleotides (ODNs). With antisense ODNs, the presumed mechanisms of action include blockage of translation, RNA transport, and splicing. The RNA-DNA hybrid can also be recognized and cleaved by RNase H, yielding an additional mechanism of repression of gene expression. Alternatively, antisense RNA generated by expression cassettes or viral vectors can block target gene expression through activation of double-stranded RNase. The first demonstration of antisense-mediated inhibition of gene expression raised considerable interest in the potential of antisense molecules not just as a molecular tool in biomedical research, but also as a novel class of therapeutic agent [9]. The molecules have been the focus of the anti-gene literature but have not succeeded in the clinic [10]. Why that is and how to make these antisense ODNs become more successful will be the subject of the next section involving the challenges to the anti-gene field [1, 10].

Future studies will be required to clarify the optimal anti-gene for each target mRNA knockout. These studies would involve a qualitative and quantitative comparison of all three anti-gene strategies in the same cell type, with the same gene target and the same RNA sequence. The anti-gene delivery system (viral or non-viral) would be appraised in different cells/tissues and *in vitro* and *in vivo*. A comparison of the rates of suppression for the target mRNA/protein and the duration of suppression would be evaluated. The consequences of these studies may reveal tissue and target mRNA specificity with different anti-gene inhibitors.

B. CHALLENGES OF ANTI-GENE STRATEGIES

With further testing of antisense oligonucleotides, a number of problems surfaced which threatened the theoretical appeal and presumed scientific mechanisms of action of these compounds. The issues of non-sequence specific and/or non-antisense activity need to be fully addressed before a particular antisense molecule is to be successfully applied to the clinic [8, 10]. Despite the many successful applications of antisense and ribozyme-based strategies, the design of such molecules remains largely empiric. Thus, proof of target gene inhibition, catalytic activity (in the case of ribozymes), sequence-specific effects (in the case of ODNs), biological activity, and the inclusion of convincing control sequences is required for each new molecule that is being tested. Nevertheless, attention to these issues will increase the possibility of a successful endeavor for anti-gene target validation and cell therapies. These issues are starting to be resolved with a better understanding of the biology and the use of more appropriate controls.

1. Gene Targets

Identifying the pivotal gene in the signal transduction pathway for a disease may or may not be easy. Some targets are obvious such as oncogenes for cancer, but these targets may not be critical in disease progression, or there may be redundancy in the signaling pathways to circumvent the gene inhibition. This has been demonstrated in several cancer model systems [1, 11]. The cell, especially human cancer cells and drug resistant cancer cells, has a myriad of responses to overcome specific gene inhibition by anti-gene technology. The half-life of the target RNA and protein may play a key role in the kinetics of the desired inhibition, especially in studies that allow for transient inhibition of the target gene. Therefore, targeting a rapidly expressing gene generally results in a more rapid phenotypic change than a gene whose protein product is more stable. Thus, increased stability of the target protein will mandate the use of stable expression systems. The inhibition of the fos-mRNA by a ribozyme in drug resistant human ovarian carcinoma cells has been shown to be one such example of mRNA upregulation and enhanced protein stability [12]. Conversely, the cell can sometimes easily circumvent the target gene inhibition by using alternate pathways or genes. This has been demonstrated in multi-drug resistant human tumor cells with the up regulation of an alternative efflux pump to eliminate cancer chemotherapeutic agents [13].

2. Target Site on the mRNA

Another important issue concerns selection of the appropriate sequence of mRNA within the target gene. The target binding sequence, secondary and tertiary RNA structure,

RNA/protein interaction and accessibility to the binding site all play a critical role in determining the efficacy of the anti-gene. This process differs from target to target and must be tested with the design of each new molecule and for each cell type. Antisense/ribozyme strategies have been successfully shown to target the 5' end of the mRNA, the 3' untranslated region, or regions in between. More important than the location within the RNA is the accessibility of the target RNA region. In this regard, the formation of stem-loops within the target mRNA as well as protein-RNA interactions may severely limit the accessibility of the target RNA to antisense ODNs or ribozymes. Thus, double stranded regions of folded single-stranded RNA and sites of interaction with RNA binding proteins are less accessible to ribozymes. In one study, a ribozyme expression library demonstrated that effective target sites for ribozymes were limited to single-stranded gaps of mRNA. Screening one human transcript for GUC and CUC triplets revealed that less than ten percent of the potential sites were accessible to ribozyme-mediated cleavage. To address this concern, RNA-folding predictions have been used in the selection of sites for ribozyme cleavage. Also, *in vitro* cleavage of target RNA can be used to screen ribozymes recognizing different sequence areas of the same gene. However, the kinetics observed with *in vitro* studies is not always predictive of ribozyme activity *in vivo*, thus underscoring the need for empiric testing [1, 14].

With respect to the design of the anti-gene molecule itself, additional factors must be considered. The first is the optimal length of anti-gene sequences. In general, most studies have used ODNs (or flanking sequences in the case of ribozymes) as short as 12-20 bases in length. However, longer antisense molecules can possess inhibitory activity. This issue may be especially relevant in designing ribozymes in view of the trade off between increased specificity (necessitating longer sequences) and improved kinetics via rapid dissociation (favored by shorter sequences). One study found a minimum length of 51 nucleotides in the 3' antisense arm to be necessary for inhibition of HIV replication [15]. Recently, a siRNA has been successfully used to target the Hepatitis B virus replication in cultured cells and in the mouse liver [16]. Conversely, short-chain ribozymes were superior when microinjected into the cytoplasm or when transcribed *in vitro*. Therefore there may be different requirements for the anti-gene length depending on the desired cellular site of inhibition.

Antisense ODNs, which contain four contiguous guanine residues (also known as G quartets), can display a high affinity for proteins and mediate anti-proliferative effects through a non-antisense mechanism. Subsequently, significant interactions between antisense ODNs and heparin binding proteins have been reported [8, 10]. ODNs can also interact in a non-sequence specific manner with the vascular endothelial growth factor (VEGF) receptor flk-1 and the epidermal growth factor receptor. In the case of flk-1, ODN binding resulted in perturbations of ligand-induced activation and receptor phosphorylation. To address these concerns, guidelines for the appropriate testing and development of specific anti-gene sequences have been established [1].

Some early successes have been noted with the siRNA technology for target gene knock down in lower organisms. However, in the more complex organisms, particularly in mammalian cells, the efficacy and therapeutics have not been as dramatic. It will require more detailed cellular studies to understand the sophisticated siRNA interactions with the target gene in these advanced and abnormally growing mammalian cells. Hopefully, the experience of the past thirty years will facilitate solving these biological challenges and efficient target knock down will be achieved. The duration of this target inhibition will also lead to a therapeutic impact on the disease tissue [17].

3. Anti-Gene Stability

Given the inherent susceptibility of DNA to serum nucleases, several modifications have been examined in order to optimize the half-life of anti-gene molecules. The most commonly used modification to the phosphodiester ODN is the phosphorothioate ODN, in which a single sulfur substitutes for oxygen at a non-bridging position at each phosphorous atom. This results in improved serum half-life but is associated with the non-antisense effects discussed previously [1, 7, 10, 14, 17]. Recently, the prototypical ODN has been defined to consist of a 20 – mer with 2'–alkyl modified bases and phosphorothioate linkages on the 5' and 3' ends of the ODN with a phosphodiester backbone. In the case of ribozymes, the modifications examined have included 2'-fluoro, 2'-amino, 2'-0-allyl and 2'-0-methyl substitutions. Another modification is the so-called chimeric ribozyme, which contains both DNA and RNA moieties. Despite encouraging results *in vitro*, extensive *in vivo* testing of these modifications has been lacking in appropriate model systems. Therefore, for *in vivo* anti-gene studies, expression of the sequence by plasmid DNA or viral vector still represents the best-tested method.

4. Delivery Systems

One of the most difficult challenges facing the anti-gene field is efficient transfer system that will stabilize, transduce and express a transgene in the target tissue. Limitations of the present vector technologies have slowed the progress of anti-gene approaches to the clinic. The strategies used to date to deliver potential antisense and ribozyme therapeutics all have significant weaknesses and at best have very narrow indications for cells and tissues. The therapeutic controversy for anti-gene strategies will always be delivery. The challenge has always been how to deliver the anti-gene: as an ODN's alone, or complexed with cationic lipids or encoded in the viral genome of the vector. The best method for delivering an anti-gene will depend on the type of tissue targeted [1, 14]. There are however some promising delivery technologies on the horizon.

The viral delivery systems have been the preferred method of introducing trans-genes into cells. The majority (~70%) of the National Institute of Health (USA) Recombinate DNA Advisory Committee approved gene therapy protocols are with viral vectors [18]. Recently, there are some controversies developing over the use of the viral vectors for gene therapy because of their ability to integrate into human chromosomes. This issue has arisen from the consequences of the gene therapy trials for the genetic disease X-linked severe combined immune deficiency. The

retroviral vector used for this gene transfer has been associated with the development of Leukemia in two patients [19]. These studies have clouded the future of viral vectors, but if these issues can be addressed, there may be opportunities for the viral vectors in some narrowly defined tissue types. Retrovirus vectors (RNA tumor viruses) and Lentiviruses have been shown to favorably transduce dividing hematopoietic cells. Adenoviruses and Adeno-Associated viruses transduce muscle and some types of tumor cells. The Herpes simplex viruses can selectively transduce nerve cells. The liver and kidney are the organs most responsible for clearing anti-genes. Conversely, this makes these organs more suitable, but more limited, as therapeutic targets. The pharmacology and toxicology of anti-gene therapy has been the rate-limiting step for efficacious impact on the disease [14]. Until more effective delivery systems are available, therapeutic doses of the anti-gene therapy will not be achieved in the disease tissue.

In the case of ribozymes expressed intracellularly, other delivery techniques have been examined, largely including viral vectors. Early studies utilized retroviral vectors, and subsequently, adenoviruses and adeno-associated viruses. Finally, in recent ribozymes studies, cationic liposome: DNA complexes have been utilized to deliver ribozymes to tumor-bearing animals [20].

Antisense ODNs and siRNA may not formally require a delivery vector [10]. As a result, these compounds have matured most rapidly to the clinical arena. Antisense ODNs are usually tested either alone or complexed with cationic liposomes. Optimizing antisense ODN delivery both to the appropriate tissues and in order to facilitate efficient intracellular uptake is of paramount importance. Uptake in non-specific tissues can lead to increased toxicity, whereas poor cellular uptake results in diminished activity. Given that antisense ODNs are polyanionic structures, they diffuse poorly across lipophilic cellular membranes. Therefore, their cellular uptake occurs mainly through absorptive and fluid-phase endocytosis. Following internalization, ODNs enter the endosomal compartment, where they are susceptible to enzymatic degradation. These delivery systems have also been used for the siRNA technology and have been successful in transducing lower organisms [2, 3, 16]. However in human model systems and *in vivo* research is more challenging for therapeutic delivery of anti-genes. These issues will be discussed in more detail in the next section.

To improve intracellular ODN delivery, multiple cations have been utilized to balance the net negative charge of ODNs. These have included cationic lipids, polylysine, polyethylenime, or transferrin-polylysine complexes. The transfection reagent cytofectin has been shown to destabilize the endosomal compartment, thereby facilitating ODN release to the cytoplasm. Specific cell or tissue targeting has been used to direct ODN uptake using receptors such as folate or transferrin.

There are some non-viral technologies being introduced to the anti-gene field using novel nanoparticle that offer several advantages over other methodologies: a diminished adverse immune response and easy manufacturing/production for the pharmaceutical industry [21]. Some of these new chemical compositions are polymers in nanometer size particles containing either DNA/stearyl polylysine coated lipids or Peptoids (DNA coated with glycine oligomers) or Cationic molecules (DNA/combined with positively charged B-cyclodextrin/adamantane and poly ethylene glycol). These molecules have been shown to be effective in cancer related angiogensis. These are promising results and if the chemists continue to collaborate with the anti-gene field, some of the issues for delivery may be overcome in the near future.

C. MODELS SYSTEMS

It is beyond the scope of this review to discuss every study utilizing anti-gene technology with potential utility to human disease. If the reader needs more comprehensive reviews the following are available for antisense [10, 22], ribozymes [12, 14] and siRNA [16]. Therefore, this discussion will focus on those targets that have shown significant therapeutic promise through vigorous testing and thus may be closest to clinical application, focusing mainly on cancer as a model system. In the realm of cancer, the following classes of genes have been targeted: (1) oncogenes; (2) cell cycle regulatory genes; (3) drug-resistance genes; (4) angiogenic genes; (5) growth factor receptor genes; and (6) genes in cell signaling pathways.

Regarding oncogenes, some of the earliest studies utilizing antisense technology targeted oncogenes with known defects in particular tumors, thus potentially representing tumor-specific agents. A prominent example included antisense targeting the bcr-abl gene formed by the chromosome 9 to 22 translocation (the Philadelphia chromosome) in chronic myelogenous leukemia (CML). Recently some patients treated with Gleevec, a first line treatment for CML, have been shown to develop resistance. The Bcr-abl fusion protein tyrosine kinase has been mutated, up regulated, or has developed altered binding properties. These events have lead to developing new therapies for CML and have become the subject of studies examining the exogenous delivery of modified ODNs [22], ribozymes [1, 14] and siRNA [16] for CML.

A second targeted approach is the use of antisense/ribozyme/siRNA targeting of the mutant *ras* gene family [1, 16, 22]. Specifically, using an antisense RNA against the entire K-*ras* gene delivered by a retroviral vector to nude mice bearing human lung carcinoma cells resulted in inhibition of tumor growth. Antisense ODNs have also been used to target mutated H-*ras*, a strategy under clinical investigation. Ribozymes against activated *ras* genes have focused on the mutated H-*ras* gene at codon 12, in which the GUC sequence in the tumor (encoding valine) is recognized by ribozyme cleavage, whereas the wild type GGC sequence (encoding glycine) is not. Delivery of anti-*ras* ribozymes has been accomplished by both retroviruses and adenoviruses [1, 22].

Additional targets for antisense therapy are comprised of genes activated in malignancies by virtue of overexpression. Prominent examples of this approach have included antisense ODNs targeting *c-myb* in CML as well as melanoma, and targeting the *c-raf-1* kinase in human bladder and breast carcinoma xenograft model [1, 22]. A somewhat related strategy has concerned the use of antisense or ribozyme approaches to inhibit expression of growth factor receptors,

including EGFR, the insulin-like growth factor I receptor, and the HER-2/*neu* receptor [1, 22].

A major use of anti-gene targeting has been to reverse resistance of tumor cells to cancer chemotherapy. The first demonstration of ribozyme targeting in cancer showed the reversal of cisplatin-drug resistance using an anti-*fos* ribozyme [12]. Subsequently, ribozymes targeting the mdr-1 gene were also utilized [13]. Antisense targeting of bcl-2 was used to chemosensitize melanomas in SCID mice [1, 22]. Finally, antisense targeting cyclin D1 has been shown to enhance drug cytotoxicity. A recent study utilizing antisense to p21 showed increased sensitivity of colon cancer cells to radiation therapy [22] and the use of siRNA targeted against the tumor suppressor genes: p53, p53 binding protein-1 and p73 [16].

Angiogenic genes comprise the next class of genes targeted by antisense/ribozymes. Ribozymes have been shown to reduce angiogenesis by targeting the VEGF receptor flt-1, the pleiotrophin gene and the gene encoding the basic fibroblast growth factor binding protein, whereas antisense technology was used to target the VEGF gene [1, 20].

Strategies to reduce metastasis represent another well-documented use of hammerhead ribozymes. This potential has been amply demonstrated in studies using ribozymes to target the CAPL/Mts 1 gene, the integrin gene α 6, the matrix metalloproteinase MMP-9, and the NF-κB pathway [1].

Finally, other targets of antisense/ribozyme therapy include cancer-associated viral genes and telomerase. Ribozymes have been shown to suppress the transformed phenotype associated with the human papillomavirus E6 and E7 genes and the Epstein-Barr Virus nuclear antigen-1. An anti-telomerase antisense construct was also linked to 2', 5'oligoadenylate and found to have additive anti-tumor activity in a glioma model when combined with adenoviral delivery of the p53 gene.

D. CLINICAL APPLICATIONS

The challenge for clinical anti-gene therapy is systemic delivery. Until this milestone is achieved, the pharmaceutical industry will not look at anti-gene molecules as a viable medical product. Progress is being made for validating these new molecular entities with the pre-clinical studies previously described using antisense ODNs and ribozymes. These compounds are now primed for clinical investigation. Currently, a number of patient studies using antisense ODNs have been completed, and many others are currently underway [22]. Administration of an antisense ODN targeting the cytomegalovirus genome (CMV) has been approved by the U.S. Food and Drug Administration for CMV-induced retinitis associated with HIV infection. Ribozymes are currently being tested in clinical trials in the setting of cancer and HIV infection [1, 18]. The speed of siRNA development should have the molecules in the clinic in the near future.

The antisense field is the furthest along in clinical studies. These studies with antisense ODNs have advanced into phase II trials and have established a safety profile for these compounds in oncological applications [1, 22]. Several dosing and administration schedules have been analyzed, and further optimization is required. Antisense ODNs have a short half-life and a fairly predictable pharmacokinetic profile [22]. Demonstration of inhibition of gene expression has been tested in a few instances in peripheral blood, tissue aspirates, and biopsy specimens, but this needs to be more conclusively examined. To date, the presence of non-antisense effects in humans is unknown. Occasional anti-tumor responses have been observed in studies utilizing antisense ODN targeting bcl-2 for lymphomas and melanomas, to *c-myb* for CML, and to protein kinase Cα for ovarian carcinomas [22]. A few phase III trials are under way, including that of the bcl-2 antisense compound G3139 in combination with dacarbazine in metastatic melanoma. Moreover, no conclusions can yet be drawn regarding the specific genes targeted [22]. Finally, the malignancies that would represent the most suitable targets for antisense therapy have yet to be defined. Nevertheless, the results amassed to date are sufficiently encouraging to permit the continued clinical maturation of antisense technology.

With respect to ribozymes, a modified chimeric ribozyme targeting flt-1 is being examined in phase II trials of solid tumors [1]. For expressed ribozymes, further optimizations in gene delivery techniques will likely be required prior to widespread clinical testing. Recent studies using systemic delivery and expression of ribozymes targeting NF-κ-B and NF-κ-B-regulated genes to reduce melanoma metastasis [20] using cationic lipid: DNA complexes with vectors that permit sustained transgene expression to tumor-bearing animals offer a possible improvement to currently existing ribozyme therapeutics for cancer.

These clinical studies have been encouraging in their lack of toxicity and safety profiles in patients, but in terms of efficacy, there is significant room for improvement. Why is there such limited success? There can be several explanations such as the following: disease indications, target selections, lack of understanding of the biology of the disease, pharmacology of these new molecular entities and systemic delivery. There is still a lot of research to be done.

SUMMARY

There have been many advances made in the field of antigene therapy over the past twenty years such as identification and validation of target genes for human diseases. Novel uses of anti-genes for therapy are emerging with potential applications to human disease. With the completion of the human genome project, a myriad of candidate genes have emerged with possible roles in the causation, prevention, and/or treatment of human disease. Target validation by anti-genes will help validate the emerging field of "Systems Biology" and the understanding of disease pathways. The use of anti-genes to suppress a target gene of interest in pre-clinical disease models may represent a powerful strategy to define the role of previously uncharacterized genes in human disease. Secondly, anti-gene technologies have been adapted to achieve inhibition or repair of mutated genes. While this represents an evolving technology at the current time, the theoretical appeal of correcting the primary genetic defects of human disease are obvious. Hopefully, we will be wiser and faster with the new communications systems for disseminating this information so that we will not need to wait another twenty years before we can impact clinic diseases.

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REFERENCES

[1] Kashani-Sabet, M. and Scanlon, K.J. (2002) in *The Cancer Handbook*, London, England: Macmillan Publisher, Chapter 91, pp. 1439-1449

[2] McManus, M. T. and Sharp, S. A. (2002). *Nature Review Genetics*, 3, 737-747.

[3] Hutvagner, G. and Zamore, P. D. (2002). *Current Opinion in Genetics & Development*, 12(2), 225-232.

[4] Zaug, A. J. and Cech, T. R. (1986) *Science*, 231, 470-475.

[5] Haseloff, J. and Gerlach, W.L. (1988) *Nature*, 334, 585-591.

[6] Herschlag, D. (1991) *Proc. Natl. Acad. Sci. USA*, 88, 6921-6925.

[7] Symonds, R. H. (1992) *Ann. Rev. Biochem.*, 61, 641-667.

[8] Stein, C. and Cheng, Y. (1993) *Science*, 261, 1004-1012.

[9] Zamecnik, P.C. and Stephenson, M.L. (1978) *Proc. Natl. Acad. Sci. USA*, 75, 280-284.

[10] Stein, C. A. (2001) *J. Clin. Invest.*, 108, 641-644.

[11] Irie, A., Kijima, H., Ohkawa, T., Bouffard, D., Suzuki, T., Curcio, L., Holm, P., Sassani, A. and Scanlon, K.J. (1997) *Adv. In Pharm.*, 40, 207-241.

[12] Scanlon, K.J., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J.J. and Kashani-Sabet, M. (1991). *Proc. Natl. Acad. Sci. USA*, 88, 10597-10595.

[13] Scanlon, K.J., Ishida, H. and Kashani-Sabet, M. (1994) *Proc. Natl. Acad. Sci. USA*, 91, 11123-11127.

[14] Scanlon, K.J. and Kashani-Sabet, M. (eds.), (1998) in *Ribozymes in the Gene Therapy of Cancer*. (R. G. Landes, Austin, TX).

[15] Sarver, N., Cantin, E. M., Chang, P. S. and Rossi, J. J. (1990) *Science*, 247, 1222-1225.

[16] McCaffrey, A. P., Nakai, H., Pandey, K., Huang, Z., Salazar, F. H., Xu, H., Wieland, S. F., Marion, P. L. and Kay, M. A. (2003) *Biotechnology*, 21, 639-644.

[17] Dykxhoorn, D. M., Novina, C. D. and Sharp, P. A. (2003) *Mol. Cell Biology*, 4, 457-467.

[18] Gottesman, M. (2003) *Cancer Gene Therapy*, 10, 501-508.

[19] Kohn, D. B., Sadelain, M. and Glorioso, J. C. (2003) *Nature Review; Cancer*, 3, 477-487.

[20] Kashani-Sabet M., Yong L. and Fong S. (2002) *Proc. Natl. Acad. Sci. USA*, 99, 3878-3883.

[21] Kirchweger, G. (2002) *Mol. Ther.*, 6, 301-302.

[22] Lebedeva, I. and Stein, C. A. (2001) *Annu. Rev. Pharmacol. Toxicol.*, 41, 403-419.